Exercise training regulates SOD-1 and oxidative stress in porcine aortic endothelium

James W. E. Rush, James R. Turk, and M. Harold Laughlin


Rush, James W. E., James R. Turk, and M. Harold Laughlin. Exercise training regulates SOD-1 and oxidative stress in porcine aortic endothelium. Am J Physiol Heart Circ Physiol 284: H1378–H1387, 2003. First published December 5, 2002; 10.1152/ajpheart.00190.2002.—Vascular oxidative stress contributes to endothelial dysfunction. Aerobic exercise training improves vascular function. The purpose of this study was to test the hypothesis that exercise training would improve the balance of antioxidant to prooxidant enzymes and reduce markers of oxidative stress in aortic endothelial cells (AEC). Female Yucatan miniature pigs either remained sedentary (SED) or were exercise trained (EX) for 16–19 wk. EX pigs had increased AEC SOD-1 protein levels and Cu/Zn SOD activity of the whole aorta compared with SED pigs. Protein levels of other antioxidant enzymes (SOD-2, catalase) were not affected by exercise training. Protein levels of p67

Address for reprint requests and other correspondence: J. W. E. Rush, Dept. of Kinesiology, BMH 1114, Univ. of Waterloo, Waterloo, Ontario, Canada N2L 3G1 (E-mail: jwerush@uwwaterloo.ca).
tive challenges (33). Adaptations in vascular endothelial cell antioxidant enzymes or indicators of oxidative stress to exercise training have not been previously reported.

Aerobic exercise training also has been reported to improve endothelium-dependent vasodilation in coronary arterioles of pigs (31), large coronary arteries of dogs (41), and in large peripheral blood vessels of humans (12, 13, 16). This adaptation may depend in part on increases in NO bioactivity, and a possible mechanism is increased NO production (i.e., increased eNOS levels and activity) after training (7, 31, 37, 45). Recent data suggest that increased vascular SOD levels may also contribute to improvements in NO bioactivity accompanying the exercise trained state (8, 35). Pig coronary arteriole SOD-1 mRNA, protein, and activity levels were all shown to increase coordinately with chronic exercise training (35). Additional data from the mouse aorta indicate that the SOD-3 protein level is elevated in response to exercise training (8). The vascular endothelium-specific (i.e., endothelium vs. blood vessel wall) adaptations in antioxidant enzymes to chronic exercise training have not been previously reported, and the impact of exercise training on vascular endothelium oxidative stress and reactive species damage to cellular and molecular components has not been evaluated. The purpose of this study was to test the hypothesis that exercise training would increase eNOS levels, improve the balance of antioxidant to prooxidant enzymes (increase antioxidant and decrease prooxidant), and reduce basal oxidative stress in vascular endothelium of pigs.

METHODS

Animals and training. Adult female Yucatan miniature swine 8–12 mo of age (n = 24) were purchased from a commercial breeder (Charles River) and housed in a room maintained at 20–23°C, with a 12:12-h light-dark cycle. All procedures involving the use of these animals were approved by the Animal Care and Use Committee at the University of Missouri. Sedentary (SED) pigs were confined to their pens during the exercise training period, whereas the exercise-trained (EX) pigs underwent a moderate aerobic exercise training regimen exactly as outlined previously (35). At week 1, pigs ran at 3 miles/h for 20–30 min, followed by 5 miles/h for 15 min, all at 0% grade. During the training period, the speed and duration of treadmill running were gradually increased such that by week 12, pigs ran for ~85 min/day, 5 day/wk. Training bouts at the beginning of week 12 and thereafter consisted of a warmup (2.5 miles/h, 5 min), a sprint (6–8 miles/h, 15 min), an endurance run (4–6 miles/h, 60 min), and a warmdown (2 miles/h, 5 min). Positive reinforcement was provided in the form of feeding after each training bout. Maximal oxygen consumption was not determined for the particular animals used in this study. On the basis of previous measurements performed in our laboratory, however, maximal oxygen consumption in similar pigs is achieved at running intensities of 10–12 miles/h (1, 21). Thus the endurance training intensity of 4–6 miles/h that the pigs in the current study performed elicited ~40–50% maximal oxygen consumption. Total duration of the exercise training program was 16–19 wk as eight pigs were managed at a time (4 SED and 4 EX pigs), and two pigs per week were euthanized over a 4-wk period (randomly selected, but 1 pig each of the SED and EX groups per week starting at week 16). Experimenters performing tissue sampling and procedures were blind to the treatment group of each pig. A period of 24 h elapsed between the last training bout and the time of death. On tissue harvesting days after the training period, SED and EX pigs were sedated with ketamine (30 mg/kg im) and anesthetized with pentobarbital sodium (35 mg/kg iv). The pigs were intubated, and ventilation was maintained with a Harvard large animal respirator throughout the tissue harvesting procedures. After an intravenous infusion of heparin (1,000 U/kg), a left thoracotomy was performed. The heart and a 15-cm piece of the thoracic aorta distal to the aortic arch were separately excised and placed in Krebs bicarbonate buffer (4°C) previously aerated with a 95% O2-5% CO2 gas mixture. Skeletal muscle samples were excised and freeze clamped at liquid nitrogen temperature. The efficacy of training was assessed by measurement of the heart weight-to-body weight ratio and by measuring citrate synthase activity in the fetal muscle (according to previously reported techniques (29)); both indexes have been previously shown to increase in EX pigs (7, 31, 37, 45).

Isolation of aortic endothelial cells. The excised aorta was cleaned of adhering tissue while submersed in a dish of Krebs buffer (4°C). A section (~3-cm-long) of the intact aorta was cut and immediately frozen (~80°C) until used for SOD enzyme activity assays. The remainder of the cleaned aorta was transferred to a fresh dish of buffer and cut longitudinally to expose the luminal surface. The tissue was then transferred to a clean glass plate, luminal surface up. Endothelial cells were harvested in one of two ways depending on the analyses that were to be performed with the harvested cells. For the immunoblot analysis, a 2-ml aliquot of TRIreagent (Molecular Research Center) was applied to the luminal surface. Thirty seconds later, this surface was scraped with the edge of a glass microscope slide, and the cell lysate was collected into a tube and immediately frozen in liquid nitrogen. For analyses of biochemical markers of oxidative stress, the luminal surface was covered in phosphate-buffered saline, and cells were scraped into centrifuge tubes using the edge of a glass microscope slide. These cell suspensions were centrifuged (2 min, 8,000 g), the supernatant was aspirated and discarded, and the cell pellets were immediately frozen in liquid nitrogen. Isolated aortic endothelial cell (AEC) preparations were tested for vascular smooth muscle cell contamination by performing immunoblots for smooth muscle α-actin (primary antibody 1:2,500, Chemicon) according to procedures outlined below in ImmunobLOTS.

ImmunobLOTS. Proteins were isolated from the TRIreagent extracts according to the manufacturer’s instructions. Isolated and washed protein pellets were dissolved in a protein solubilization buffer consisting of 50 mM Tris·HCl (pH 7.4), 6 M urea, and 2% SDS. Protein concentration was determined using the bicinchoninic acid assay (Pierce). Before electrophoresis, aliquots of these samples were supplemented with 150 mM dithiothreitol and boiled for 1 min. Samples containing 30 μg protein were loaded onto gels and electrophoresed. Proteins were subsequently electroblotted to polyvinylidene difluoride membranes. Proteins of interest were detected by immunoblotting using primary antibodies specific for SOD-1 (1:2,500, Stressgen), SOD-2 (1:2,500, Stressgen), catalase (1:5,000, Chemicon), eNOS (1:2,500, Transduction Labs), p47phox (1:1,000, Chemicon), and p67phox (1:5,000, Chemicon). Secondary antibodies were conjugated with horseradish peroxidase. All antibody and blocking solutions contained 5% nonfat milk and 0.1% Tween 20 in Tris-buffered saline. Chemiluminescent signals (ECL, Amer-
SOD enzyme activity assays. A previously used (35) SOD activity assay based on the inhibition of autooxidation of pyrogallol was used to determine SOD activity in the intact aorta (not AEC preparations). Ring segments cut from the intact aorta were weighed and homogenized in 9 vol of 50 mM Tris-citric acid (pH 8.2) containing 1 mM diethylenetriaminepentaacetic acid (DPTA) using a ground glass homogenizer, followed by a 15-s burst of sonic oscillation. For both homogenization and sonication, the tube containing the sample was immersed in an ice slurry to dissipate heat. The homogenate was centrifuged (1,000 g, 5 min, 4°C) to pellet cell debris, and the supernatant was used for assays. An aliquot of untreated supernatant was used for total SOD activity assays, whereas Cu/Zn-specific SOD assays were performed on a separate aliquot of supernatant that was additionally extracted in ethanol-chloroform [62:5:37.5 (vol/vol)] to inhibit Mn-dependent SOD activity and therefore to make the assay specific for CuZn SOD (35). Assays were performed in a total volume of 1 ml consisting of 800 µl of 50 mM Tris-citric acid, 1 mM DPTA (pH 8.2) equilibrated with room air at 25°C, and 100 µl aorta homogenate, and the reaction was initiated by the addition of 100 µl of 2 mM pyrogallol (final concentration = 0.2 mM). The increase in absorbance at 420 nm was followed for 10 min. SOD activity was assessed as the degree of inhibition of the pyrogallol autooxidation rate. The autooxidation rate (absence of homogenate) under the indicated conditions was 0.0202 ± 0.0002 optical density units/min, n = 20, assessed over the first 3 min of the reaction. This assay system yielded proportional results when between 50 and 100 µl of homogenate were used and the reaction rate was linear for up to 10 min. Values were averaged over the first 3 min of the reaction. One unit of SOD activity is defined as the amount of activity that inhibits the autooxidation rate of pyrogallol by 50%. Data are expressed as units per gram of tissue.

Markers of oxidative stress. Malondialdehyde (MDA) levels were determined as an index of lipid peroxidation using the Calbiochem lipid peroxidation assay kit. This method takes advantage of the formation of a stable chromophore (maximal absorbance at 586 nm) between MDA and 4,5-dihydroxyacetophenone (DHPA) and 2-thiobarbituric acid (TBA) and is based on the thiobarbituric acid reaction. Platelet suspensions in saline, after exposure to different concentrations of oxygen, were used as a source of MDA. The buffer and water wash steps were performed after each protocol step. Sections were incubated with avidin-biotin two-step blocking solution (Vector SP-2001) to inhibit background staining and 3% hydrogen peroxide to inhibit endogenous peroxidase. Nonimmunoperoxidase labeling protocol was applied to inhibit nonspecific protein binding. Primary antibodies were added and incubated overnight at 4°C. The primary antibodies used were rabbit polyclonal anti-nitrotyrosine (Chemicon) at 1:1,000 dilution or rabbit polyclonal anti-MDA (ABCAM) at 1:200 dilution. After the appropriate washing steps were completed, the sections were incubated with biotinylated anti-rabbit link secondary antibody in phosphate-buffered saline containing 15 mM sodium azide and peroxidase-labeled streptavidin (Dako LSAB+ kit, peroxidase, K0690). Diaminobenzidine (Dako) applied for 5 min allowed visualization of primary antibody staining and was followed by diaminobenzidine enhancer (Dako S1961). Sections were counterstained with Mayer's hematoxylin stain for 1 min, dehydrated, and coverslipped. For negative controls, histological sections were prepared as described above, but incubation in primary antibody was excluded from the protocol. Sections were examined and photographed using an Olympus BX40 photomicroscope. Semiquantitative analysis of immunohistochemical images was performed using Image-Pro Plus software (Media Cybernetics) to integrate the positive brown pixel count over the AEC portion of the images.

In addition to these biochemical and immunohistochemical markers of oxidative stress, a stress-sensitive signaling pathway, the extracellular signal-regulated kinase (ERK-1/2) pathway, was evaluated. Activation of this pathway was assessed via immunoblot analysis of phosphorylated and total ERK-1/2 content in AEC. These analyses were performed using rabbit polyclonal antibodies specific for either total ERK-1/2 (Cell Signaling Technology, 1:1,000) or for phospho-ERK-1/2 (Cell Signaling Technology, 1:1,000). Relative protein levels of the oxidative stress-sensitive heme oxygenase (HO-1) were determined by immunoblotting using a rabbit polyclonal antibody (Stressgen, 1:500).

**RESULTS**

**Efficacy of training.** As illustrated in Table 1, the heart weight was ~15% higher (P = 0.01) and the heart weight-to-body weight ratio was ~10% higher (P = 0.04) in EX vs. SED pigs, whereas the body weight was ~14.6% lower (P = 0.01). The heart weight/body weight ratio was ~2% higher (P = 0.01) in EX vs. SED pigs, whereas the body weight was ~14.6% lower (P = 0.01). The heart weight/body weight ratio was ~2% higher (P = 0.01) in EX vs. SED pigs, whereas the body weight was ~14.6% lower (P = 0.01). The heart weight/body weight ratio was ~2% higher (P = 0.01) in EX vs. SED pigs, whereas the body weight was ~14.6% lower (P = 0.01). The heart weight/body weight ratio was ~2% higher (P = 0.01) in EX vs. SED pigs, whereas the body weight was ~14.6% lower (P = 0.01). The heart weight/body weight ratio was ~2% higher (P = 0.01) in EX vs. SED pigs, whereas the body weight was ~14.6% lower (P = 0.01). The heart weight/body weight ratio was ~2% higher (P = 0.01) in EX vs. SED pigs, whereas the body weight was ~14.6% lower (P = 0.01). The heart weight/body weight ratio was ~2% higher (P = 0.01) in EX vs. SED pigs, whereas the body weight was ~14.6% lower (P = 0.01). The heart weight/body weight ratio was ~2% higher (P = 0.01) in EX vs. SED pigs, whereas the body weight was ~14.6% lower (P = 0.01).

**Table 1. Indexes of efficacy of the aerobic training program**

<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Exercise Trained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight, g</td>
<td>156.5 ± 6.7</td>
<td>177.3 ± 3.6*</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>34.2 ± 0.9</td>
<td>35.4 ± 0.9</td>
</tr>
<tr>
<td>Heart weight/bod weight, g/kg</td>
<td>4.6 ± 0.2</td>
<td>5.0 ± 0.2*</td>
</tr>
<tr>
<td>Deltid citrate synthase activity, mmol·min⁻¹·g⁻¹</td>
<td>14.6 ± 0.3</td>
<td>18.7 ± 1.3†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 animals/group. *P < 0.05 and † P < 0.01, significantly greater than corresponding values for the Sedentary group.
was not significantly different between SED and EX pigs \( (P = 0.18) \). In addition, citrate synthase activity of the deltoid muscle increased by \(~ 30\% \) as a result of aerobic exercise training (Table 1). These data support numerous other studies that have demonstrated the efficacy of this exercise regimen in eliciting stable cardiovascular and skeletal muscle training adaptations (22, 31, 35).

**Purity of the AEC preparations.** To confirm that the cell preparations prepared by scraping the luminal surface of the aortas were not contaminated with vascular smooth muscle cells, immunoblots were performed using an antibody to vascular smooth muscle actin (Chemicon, 1:2,000). Although actin was readily detected in the positive control whole aorta preparations (Fig. 1, lane 5, \(~ 42 \text{kDa} \) band), there was no detectable signal in the endothelial cell lanes of this blot even when exposed for 10 min. There was no detectable signal in the endothelial cell lanes of this blot even when exposed for 10 min, the longest exposure used.

**AEC protein adaptations to exercise training.** The relative SOD-1 protein level assessed by immunoblot was increased by \(~ 90\% \) in EX vs. SED AEC \( (P = 0.009; \text{Fig. 2, } \sim 17-\text{kDa band}) \), whereas a small \((\sim 17\% \) increase in SOD-2 protein in response to EX was not statistically significant \( (P = 0.164, \text{Fig. 2; } \sim 22 \text{kDa band}) \). The catalase protein level was not significantly different in EX vs. SED AEC \( (P = 0.317; \text{Fig. 2, } \sim 62-\text{kDa band}) \). Although the levels of the two subunits of NAD(P)H oxidase, p47phox and p67phox, were each reduced by \(~ 25-30\% \) in response to training, this was not statistically significant for p47phox \( (P = 0.273; \text{Fig. 2, } \sim 47-\text{kDa band}) \) but was for p67phox \( (P = 0.050; \text{Fig. 2, } \sim 67-\text{kDa band}) \). The protein level of eNOS assessed by immunoblot was not different in EX vs. SED AEC preparations \( (P = 0.438; \text{Fig. 3, } \sim 140-\text{kDa band}) \).

**Aorta SOD activity adaptations to exercise training.** Total (Cu/Zn dependent + Mn dependent) SOD activity in homogenates of intact aortic rings was not significantly affected by exercise training \( (P = 0.153) \). However, the \(~ 25\% \) increase in aorta Cu/Zn-dependent SOD activity in EX vs. SED pigs was statistically significant \( (P = 0.049; \text{Fig. 4}) \). Mn-dependent SOD activity, determined as the difference between total SOD and Cu/Zn-dependent SOD activities, was not significantly affected by exercise training \( (P = 0.277; \text{Fig. 4}) \).

**Oxidative stress marker adaptations to exercise training.** Nitrotyrosine content in AEC proteins assessed by integrated scanning densitometry of the five most detectable bands from nitrotyrosine immunoblots (Fig. 5) was not different in EX vs. SED pigs \( (0.979 \pm 0.080 \text{ vs. } 1.000 \pm 0.046 \text{ relative density units, respectively, } P = 0.414) \). Similarly, immunohistochemical analysis of endothelial cell nitrotyrosine content revealed no significant effect of exercise training (Fig. 5). Lipid peroxidation in AEC, indexed by biochemical analysis of MDA content, was decreased in EX vs. SED AEC \( (0.7 \pm 0.1 \text{ vs. } 1.3 \pm 0.2, P = 0.017, n = 4 \text{ and } 6 \text{, respectively}) \). Similarly, immunohistochemical analysis revealed a decreased endothelial cell MDA content in EX vs. SED AEC (Fig. 6).

Total ERK-1/2 protein level in AEC was not affected by exercise training \( (P = 0.359; \text{Fig. 7}) \), whereas phos-
pho-ERK-1/2 was significantly decreased in EX vs. SED AEC (P = 0.001; Fig. 7). ERK-1/2 consists of two distinct proteins: p44 and p22 MAPK. Thus ERK-1/2 is detected as two bands in immunoblots: one at 44 kDa (ERK-1) and the other at 42 kDa (ERK-2). For quantitative purposes, the 44- and 42-kDa bands were combined. Expressed as the ratio of phospho-ERK-1/2 to total ERK-1/2, calculated on an individual basis for each different pig, the phospho-ERK-1/2 fraction was significantly decreased by exercise training (P = 0.023; Fig. 7). HO-1 protein level in AEC was not significantly affected by exercise training (P = 0.175; Fig. 7, ~32-kDa band).

**DISCUSSION**

The major findings of this study are that long-term aerobic exercise training of pigs increases SOD-1 protein levels in the vascular endothelium of the aorta and that functionally this adaptation is associated with higher aorta SOD-1 enzyme activity levels, lower biomolecular indexes of oxidative stress (lower MDA concentrations), and decreased activation of the stress-sensitive ERK-1/2 signaling pathway. The protein levels of other antioxidant enzymes (SOD-2, catalase) in the aortic endothelium are not altered compared with SED animals in these long-term trained pigs. The increased levels of SOD-1 are expected to better manage O$_2^-$ and improve NO bioavailability. There was no significant change in the eNOS protein level in AEC with long-term exercise training in this study. Thus improved management of oxidative stress may be a major mechanism of exercise-induced improvements in NO bioactivity. The data reported herein are the first to assess endothelial-specific exercise training adaptations in antioxidant enzymes and to quantify markers of oxidative stress in vascular endothelial cells directly harvested from animals.

It has previously been reported that exercise training increases aortic eNOS levels in the dog aorta (37). In the dog study of Sessa and colleagues (37), however, exercise training was for only 10 days. Thus the reported increase in eNOS in the aorta of these animals may have been a transient phenomenon that occurred in the early phase of training and may not persist in the steady-state trained phenotype. The lack of stable adaptation in eNOS levels in large-caliber arteries in response to prolonged aerobic exercise training reported here is supported by recent findings of no change in eNOS protein levels in the left anterior descending conduit coronary arteries of EX pigs (22). It is important to appreciate this apparent training-duration distinction in large artery eNOS adaptations, because improvements in vascular endothelial function accompanying exercise training have been ascribed to increased eNOS based chiefly on short-term training data. Data from the current study suggest that adaptations in the enzymes controlling vascular oxidative stress should also be considered.

There have been few previous studies of vascular cell antioxidant enzyme adaptations to exercise training. Fukai and colleagues (8) reported an increased SOD-3 protein level and no change in SOD-1 protein level in the mouse aorta after 3 wk of moderate intensity treadmill exercise. However, enzyme activity measurements were not made, so the functional significance of possible increases in SOD-3 with training is uncertain at present. In addition, the degree of cardiovascular adaptation that had occurred in the EX mice cannot be ascertained, because no indexes of training adaptations were reported (8). Thus it is not possible to determine whether these were steady-state adaptations or transient phenomena occurring during the early phase of training (training duration was only 3

![Fig. 3. Immunoblot analysis of the exercise training effect on endothelial nitric oxide synthase (eNOS) protein levels in AEC. Top: representative luminograms of eNOS using n = 4 samples/treatment group, each from a different animal. Bottom: densitometric analysis of immunoblots. Data are expressed as means ± SE. This graph represents composite data from n = 6 animals/treatment group.](http://ajpheart.physiology.org/)

![Fig. 4. Enzyme analysis of the exercise training effect on superoxide dismutase (SOD) activity in the aorta. Data are expressed as means ± SE; n = 4 samples/treatment group, each from a different animal. Cu/Zn, Cu/Zn-dependent SOD activity; Mn, Mn-dependent SOD activity; total, Cu/Zn-dependent + Mn-dependent SOD activity. One unit is the amount of SOD activity that inhibits the autooxidation rate of pyrogallol by 50%. *P < 0.05, SED vs. EX AEC.](http://ajpheart.physiology.org/)
Fig. 5. Immunoblot and immunohistochemical analysis of the exercise training effect on protein nitrotyrosine in the aorta. A, left: immunoblot analysis of AEC; n = 4 samples/treatment group, each from a different animal. Scanning densitometry of the five most detectable bands revealed relative nitrotyrosine levels of 0.979 ± 0.080 vs. 1.000 ± 0.046 relative density units in EX vs. SED AEC samples (P = 0.414). Right, immunohistochemical analysis of aorta sections (representative images). Arrows, endothelium; area below arrows, aorta media. Bar = 100 μm.

B: semiquantitative analysis of the SED vs. EX effect on nitrotyrosine; n = 6 samples/group, each from a different animal.

Fig. 6. Immunohistochemical analysis of the exercise training effect on lipid peroxidation in the aorta. A: representative images of malondialdehyde (MDA) in the wall of the aorta. Arrows, endothelium; area below arrows, aorta media. Bar = 100 μm. B: semiquantitative analysis of the SED vs. EX effect on MDA; n = 6 samples/group, each from a different animal. *P < 0.05, SED vs. EX.
Fig. 7. Immunoblot analysis of the exercise training effect on ERK-1/2, phospho-ERK-1/2, and heme oxygenase (HO)-1 protein levels in AEC. Top: representative luminograms of total ERK-1/2, phospho-ERK-1/2, and HO-1 using \( n = 3 \) samples/treatment group, each from a different animal. Bottom: densitometric analysis of immunoblots for each protein. Data are expressed as means ± SE. This graph represents composite data from \( n = 3 \) animals/treatment group, each analyzed in duplicate. *\( P < 0.05 \) and †\( P < 0.01 \), SED vs. EX AEC.

It is likely that the vascular endothelial adaptation in SOD-1 reported here is a characteristic of the stable exercise-trained state but may not be realized early in exercise training. We have previously reported that long-term aerobic exercise training, which results in steady-state cardiovascular and skeletal muscle training adaptations in pigs, is associated with increases in SOD-1 mRNA, protein, and enzymatic activity levels in coronary arterioles (35). Thus there is precedent for an exercise-induced increase in vascular SOD-1. Importantly, however, the current study demonstrates that training-induced increases in SOD-1 can occur in endothelial cells (previous studies with the aorta or arterioles could not distinguish between endothelial and vascular smooth muscle adaptations) and that functionally this is associated with indexes of lower endothelial cell oxidative stress. In our previous work (35), we reported no change in porcine coronary arteriole SOD-2 and catalase levels with exercise training, results that are supported by the current data using isolated AEC. Although the GPx/glutathione reductase couple is part of the cellular enzymatic antioxidant defense system, it was not studied in the current investigation due to limitations in tissue availability and reagents. Thus assessment of the importance of this system in the vascular endothelium exercise training response cannot be made at present.

Enzymatic activity of SOD was determined using whole aorta homogenates. Thus endothelial, vascular smooth muscle, and extracellular compartments, all of which contain SOD, are included in this analysis. Nonetheless, the enzyme activity data support the SOD protein levels detected using immunoblots of isolated AEC; Cu/Zn SOD activity was higher in EX vs. SED aortas, whereas SOD-2 activity was not affected by training. We cannot say from our data that the increase in Cu/Zn-SOD activity occurred exclusively in the endothelial cells, but this would be consistent with the \( \sim 90\% \) increase in SOD-1 protein in the AEC of EX vs. SED animals. The smaller relative exercise-induced increase in aorta Cu/Zn-SOD enzymatic activity (\( \sim 25\% \)) compared with the AEC SOD-1 protein level could be due to the fact that endothelial cells account for a small fraction of total aortic tissue. We did not specifically assess possible SOD-3 enzyme activity responses to training in this study and thus cannot report possible contributions of SOD-3 to the Cu/Zn-SOD activity data. Significant contribution of SOD-3 to the SOD enzyme activity results is unlikely under our experimental conditions; however, because the pigs were treated with 1,000 U/kg heparin before tissue harvesting. This dose of heparin has previously been demonstrated to result in a maximal release of SOD-3 from the extracellular matrix of the blood vessel wall to the plasma (18).

In the current study, there was a small but marginally significant reduction in \( p67^{phox} \) in the isolated AEC preparation from EX vs. SED pigs. Studying adaptations in vascular NAD(P)H oxidase is complicated by the fact that it is a multisubunit complex composed of cytosolic components (\( p47^{phox}, p67^{phox}, \) and \( \text{Rac1} \)) as well as membrane-spanning components (\( \text{gp91}^{phox} \) and \( \text{p22}^{phox} \)), which together comprise flavocytochrome \( b_{558} \) (10). Physical and chemical stimuli that result in NAD(P)H-dependent \( \text{O}_2^- \) generation induce association of the cytosolic and membrane-bound components and activation of the enzyme complex (10, 11). Thus, although useful information is provided by determining the adaptations in one or multiple subunits of the NAD(P)H complex, the possibility of a noncoordinated response in the expression of other subunits, an alteration in the assembly of the complex in response to stimuli, and other regulatory features that affect the flux through the fully assembled enzyme complex also influence the \( \text{O}_2^- \) production by this enzyme and could potentially contribute to altered regulation of this enzyme in response to perturbations like chronic exercise training. In the absence of data examining inducible NAD(P)H oxidase-derived \( \text{O}_2^- \) production in AEC from SED and EX pigs, no specific conclusions regarding the possible functional impact of exercise training on NAD(P)H oxidase can be made from the current data.

With an increase in the antioxidant enzyme SOD-1, it could reasonably be expected that cellular biochemical markers of oxidative stress would be reduced in the
AEC of EX vs. SED pigs. Reduction in MDA concentration, detected by both biochemical and immunohistochemical techniques, indicates less lipid peroxidation damage in EX vs. SED AEC. Nitrotyrosine levels, detected by both immunoblot and immunohistochemical techniques, were unchanged, implying no change in the net peroxynitrite-induced protein modifications. This may be expected in light of the lack of change in AEC eNOS even though the SOD-1 level was increased, because the rate constant for NO-O₂⁻ interaction is so rapid (4, 5). Activation of ERK-1/2 in endothelial cells has previously been shown to be redox sensitive (10, 46). Thus the observation of reduced phospho-ERK-1/2 in EX vs. SED AEC supports the biochemical data indicating a lower basal oxidative stress in AEC after training. It has been established that an increase in HO-1 expression occurs with increases in oxidative stress (6) in multiple cell types, including endothelial cells (6). It is less clear whether a decrease in the HO-1 level can be expected to result from a decrease in oxidative stress below normal basal conditions. Thus it is uncertain whether the lack of change in the HO-1 protein level is consistent with other observations implicating a reduction in oxidative stress in EX vs. SED AEC.

Increased vascular oxidative stress and endothelial dysfunction are associated with a number of cardiovascular disease risk factors including atherosclerosis, hypertension, and diabetes (4, 10, 20). Furthermore, regular aerobic exercise training is associated with at least partial recovery of endothelial function in human hypertensive and chronic heart failure patients (12, 13, 16). The results of this study provide a possible mechanistic basis for the exercise training effect; increased vascular antioxidant capacity, decreased oxidative stress, and improved NO bioactivity. Even in humans or experimental animals not suffering from frank endothelial dysfunction, exercise training has been shown to have beneficial effects on endothelial function (7, 16, 31, 41). Thus it is possible even in seemingly healthy but sedentary individuals that exercise training-induced improvements in vascular antioxidant enzyme levels and reduced oxidative stress could improve NO-dependent function. It is not clear whether reductions in oxidative stress are necessarily beneficial to the cell in a more global sense because redox-dependent regulation is a mechanism of normal cell signaling and gene expression (2).

In this study, only female pigs were used. It is recognized that gender differences in vascular function exist that seem to additionally depend on the drug/agonist used to elicit the test response, the species of animal studied, and the anatomic origin of the artery (23, 27). This includes observations of greater endothelium-dependent dilation and basal NO release in female compared with male arteries (14, 15). Furthermore, gender differences in functional adaptations of large peripheral arteries to exercise training have been reported (23). Some of the gender-dependent vascular effects may be due in part to the genomic and non-genomic effects of estrogen. For instance, estrogen potentiates endothelium-dependent vasodilation acutely (3) and also influences the expression of eNOS (26, 43). Because of these recognized gender-dependent effects, a limitation of the current study is the possibility that the female data presented in this study may not apply to males. The existence of gender-dependent exercise training adaptations in these systems is a possibility because gender differences in vascular endothelial prooxidant and antioxidant systems have not been reported in experimental animals or humans.

The mechanisms accounting for selective increases in SOD-1 in AEC of EX animals are not known. SOD-1 is known to be a shear stress-sensitive gene product (17). It is possible that the repeated increased flow/shear accompanying increased cardiac output during exercise training bouts contributes to the ultimate increased expression of SOD-1 in AEC. In addition, it is possible that the local generation of ROS in vascular cells during bouts of exercise trigger the cellular events leading to increased SOD-1 expression. Thus redox-sensitive transcription factors including specificity factor 1, activator protein-1, and nuclear factor-κB may be involved in a cascade of events resulting in the observed adaptations (2). This study cannot distinguish between these and other possible mechanisms accounting for the results.

In summary, prolonged, regular aerobic exercise training increased SOD-1 protein in AEC and Cu/Zn-dependent SOD activity in the aorta of pigs. There is evidence for a decrease in some subunits of the major vascular cell prooxidant enzyme NAD(P)H oxidase in EX vs. SED AEC. Protein levels of other antioxidant enzymes and of eNOS were not different in AEC of EX vs. SED pigs. The noted adaptations were accompanied by indexes of lower oxidative stress, including a decrease in the lipid peroxidation product MDA and a decreased activation of the ERK-1/2 signaling pathway but not by changes in HO-1 protein levels. These data suggest that ROS management and therefore NO bioactivity is improved in EX animals. This could contribute to improved endothelial function and cardiovascular health of individuals who regularly exercise compared with their sedentary counterparts.

We acknowledge the outstanding technical assistance of Lisa Code, Denise Holiman, Tammy Strawn, and Pam Thorne.

This research was funded by Heart and Stroke Foundation of Ontario Grant NA-4604 (to J. W. E. Rush) and by National Heart, Lung, and Blood Institute Grant HL-52490 (to M. H. Laughlin).

REFERENCES


H1386 ENDOTHELIAL OXIDATIVE STRESS AND EXERCISE TRAINING


